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Control of Glycolysis in Cerebral Cortex Slices

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1. Intracellular concentrations of intermediates and cofactors of glycolysis were measured in guinea-pig cerebral cortex slices incubated under varying conditions. 2. Comparison of mass-action ratios with apparent equilibrium constants for the reactions of glycolysis showed that hexokinase, phosphofructokinase and pyruvate kinase catalyse reactions generally far from equilibrium, whereas phosphoglucose isomerase, aldolase, phosphoglycerate kinase, phosphoglycerate mutase, enolase, adenlyate kinase and creatine phosphokinase are generally close to equilibrium. The possibility that glyceraldehyde 3-phosphate dehydrogenase may catalyse a 'non-equilibrium' reaction is discussed. 3. Correlation of changes in concentrations of substrates for enzymes catalysing 'non-equilibrium' reactions with changes in rates of glycolysis caused by alteration of the conditions of incubation showed that hexokinase, phosphofructokinase, pyruvate kinase and possibly glyceraldehyde 3-phosphate dehydrogenase are subject to metabolic control in cerebral cortex slices. 4. It is suggested that the glycolysis is controlled by two regulatory systems, the hexokinase–phosphofructokinase system and the glyceraldehyde 3-phosphate dehydrogenase–pyruvate kinase system. These are discussed. 5. It is concluded that the rate of glycolysis in guinea-pig cerebral cortex slices is limited either by the rate of glucose entry into the slices or by the hexokinase–phosphofructokinase system. 6. It is concluded that addition of 0·1 mM-ouabain to guinea-pig cerebral cortex slices causes inhibition of either glyceraldehyde 3-phosphate dehydrogenase or phosphoglycerate kinase or both, in a manner independent of the known action of ouabain on the sodium- and potassium-activated adenosine triphosphatase.

Correlation of changes in rates of flow through metabolic pathways with changes in steady-state concentrations of intermediates provides the most direct means of investigating the control of metabolism. Previous studies of the control of glycolysis in brain have analysed mainly the effects of ischaemia on the intact brain (Thorn, Scholl, Pfleiderer & Mueldener, 1958; Lowry, Passonneau, Hasselberger & Schulz, 1964; Lowry & Passonneau, 1964). It is the purpose of this work to investigate the control of glycolysis in aerobically incubated cerebral cortex slices. Intracellular concentrations of intermediates of glycolysis in guinea-pig cerebral cortex slices have been measured under conditions giving different rates of glycolysis. Two preliminary communications of some of this work have appeared (Rolleston & Newsholme, 1966*a*,*b*).

In this paper, regulatory enzymes are defined as those whose activities may be controlled by factors other than the supply of their substrates, and can thus control metabolic pathways. The term regulatory is used to include enzymes whose activities control the concentrations of intermediates within a pathway as well as those controlling rates of flow.

In interpretation of the measurements made in this work, the reactions of glycolysis were first classified according to their displacement from equilibrium, on the premise that only those enzymes that catalyse reactions far from equilibrium can control metabolic pathways (e.g. Mann, Trevelyan & Harrison, 1958; Hess, 1963; Bücher & Rüssmann, 1964). Those reactions far from equilibrium ('non-equilibrium') were then tested for their regulatory significance by using the premise that a change in the concentration of substrate for a 'non-equilibrium' reaction in the opposite direction to the change in rate of flow through that reaction indicates that the enzyme concerned is regulatory (Krebs, 1957). Since this test is applied only to 'non-equilibrium' reactions, changes in product concentrations do not affect these conclusions; consequently it is not necessary to demonstrate a

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'crossover-point' (see Chance, Holmes, Higgins & Connelly, 1958) to conclude that a 'non-equilibrium' enzyme is regulatory. For the same reason, changes of mass-action ratios or of ratios of intermediates do not of themselves permit 'non-equilibrium' enzymes to be identified as regulatory. This is discussed more fully by Rolleston (1966).

MATERIALS AND METHODS

Unless otherwise stated, materials and methods were as described by Rolleston & Newsholme (1967).

Materials. Aconitase was prepared from pig heart by the methods of Morrison (1954) and Siebert (1963) with tricarballylic acid instead of citric acid used to stabilize the enzyme (Golberg, Passonneau & Lowry, 1966). All other enzymes not already described (Rolleston & Newsholme, 1967) were obtained from Boehringer Corp. (London) Ltd. Inulin was obtained from British Drug Houses Ltd., Poole, Dorset. Vilene is a common dress-making material.

Methods. Measurements of rates of glucose metabolism and oxygen consumption have been described (Rolleston & Newsholme, 1967). The concentrations of potassium in the media were varied by interchanging potassium and sodium in the Krebs & Henseleit (1932) saline.

In experiments with washed slices, the weighed tissue samples were shaken in 6ml. of potassium-free saline containing 5mM-glucose for 1hr. at 0° by the method of Elliott & Bilodeau (1962). These slices were separated from the washing medium by filtration under gentle suction through Vilene supported on sintered glass, and transferred to the incubation flasks. For measurement of intermediates, slices were incubated in 25ml. Erlenmeyer flasks containing 3ml. of medium (Krebs & Henseleit, 1932). Incubations were ended by a process designed to cool the tissue to approx. −190° and deproteinize the incubation medium at 0° as rapidly as possible. The tissue slices were removed from flasks from the medium with forceps and crushed between two aluminium plates cooled in liquid nitrogen; the medium was filtered through Vilene and into 60% $HClO_4$ (final concn. 6%) at 0°. Freezing of the flask contents (up to three pieces of tissue) could be completed within 5sec. from opening the flask, and deproteinization of the medium in a further 5sec. The contents of up to six flasks (150mg. of fresh tissue/flask) were pooled to obtain enough tissue for extraction.

The frozen tissue was weighed and extracted by grinding with 3ml. of 50% acetone containing 12% $HClO_4$ and 5mM-EDTA in a porcelain mortar cooled in liquid air. The resultant paste was transferred to a weighed, graduated centrifuge tube, and stored in solid CO_2 until all samples were ready. Each sample was homogenized while thawing (approx. −20°). The tissue and medium samples were centrifuged at 1500*g* for 15min. and the supernatants decanted into graduated centrifuge tubes. Samples were taken for phosphate and inulin determinations, and the remainders of the extracts were brought to pH6 with 30% KOH saturated with KCl, by using an internal indicator. The tubes were left on ice for 30min. and centrifuged at 1000*g* for 10min. to remove the precipitated $KClO_4$. The supernatants were decanted and used for assays of intermediates.

The pellets of precipitated tissue solids obtained on extraction were washed once with aq. 2% $HClO_4$, dried overnight at 105° and then for a further 4hr. at room temperature over $CaCl_2$ at reduced pressure, and weighed. The dry weights obtained were assumed to represent the dry weight of the tissue extracted; control experiments showed that the error inherent in this assumption was less than 5%.

Analyses. Inorganic phosphate was measured by the method of Berenblum & Chain (1938) as modified by Martin & Doty (1949). Inulin was measured as described by Varon & McIlwain (1961). The remaining compounds were assayed enzymically; methods were taken from Bergmeyer (1963). Tissue extracts were analysed for all compounds reported; incubation media were analysed only for those compounds that were detectable in them, e.g. glucose, lactate, pyruvate, triose phosphates, creatine, inorganic phosphate and inulin. Measurements of intermediate concentrations are expressed as mμmoles/ml. of intracellular fluid with inulin used as an extracellular marker (Pappius & Elliott, 1956; Varon & McIlwain, 1961). However, in analysis of the changes of intermediates with time (Table 2) results were expressed as mμmoles/g. dry wt. as the shorter times would not have permitted inulin to equilibrate with the extracellular space (Varon & McIlwain, 1961). In Tables 3 and 4, multiplication of the concentrations of intermediates by the conversion factors given (ml. of intracellular fluid/g. dry wt. of tissue) permits the results to be expressed as amounts.

RESULTS

Effects of glucose concentration, potassium, cyanide, glutamate, aspartate and ouabain on rates of glucose utilization and lactic acid formation

In all incubations, cerebral cortex slices incubated in the control condition (5·9mM-potassium) were compared with the remaining conditions analysed. In confirmation of earlier workers, the addition of glutamate (Weil-Malherbe, 1938), aspartate, cyanide (see McIlwain, 1966), 0·1mM-ouabain (Wollenberger, 1947; Schwarz, 1962; Swanson & McIlwain, 1965) or increasing the concentration of potassium (Ashford & Dixon, 1935) caused increased rates of glucose utilization and lactate formation in cerebral cortex slices (Table 1). 1μM-Ouabain caused decreased rates of glucose utilization and lactate formation without affecting oxygen consumption (Table 1). In slices incubated in 50mM-potassium or in the presence of cyanide, increasing the concentration of glucose from 5 to 10mM caused increased rates of glucose uptake and lactate formation (Table 1); this did not occur in slices incubated in 5·9mM-potassium, or in the presence of glutamate, aspartate or 0·1mM-ouabain.

The effects of the absence of potassium were investigated with slices that had been washed to reduce their content of potassium. In these washed slices, glucose utilization and lactate formation were higher in the absence of potassium than in

Table 1. *Effects of potassium, glutamate, aspartate, cyanide and ouabain on glucose utilization and lactate formation by guinea-pig cerebral cortex slices at varying concentrations of glucose*

Slices were incubated as described in the Materials and Methods section. Results are expressed as means ± S.D. of four independent observations. (Slices incubated in 5·9mM-potassium were analysed in each incubation, and these results are averaged from 24 independent observations.) * ($P < 0.01$), Significance of differences from control experiments; ** ($0.01 < P < 0.05$), significance of differences from slices incubated at 5mM-glucose but otherwise under the same conditions.

Concn. of K^+ ion (mM) Fresh slices	Additions	Concn. of glucose (5mM)	Rate (μmoles/g. of fresh tissue/30min.) Glucose utilization	Lactate formation
5·9	None	5 or 10	10·9±0·9	6·1±3·2
5·9	Glutamate (5mM)	5 or 10	13·7±1·0*	15·5±2·3*
5·9	Aspartate (5mM)	5 or 10	14·7±3·0*	10·2±1·6*
5·9	Ouabain (0·1mM)	5 or 10	18·3±2·8*	20·5±2·5*
5·9	Ouabain (0·001mM)	5 or 10	5·9±0·9*	4·1±5·6
5·9	Cyanide (3mM)	5	44·0±6·8*	84·6±5·8*
5·9	Cyanide (3mM)	10	57·0±9·3	108·9±14·1**
5·9	Cyanide (3mM)	50	—	115·3±16·5
50	None	5	28·2±4·5*	32·8±5·9*
50	None	10	41·0±6·9**	59·9±7·2**
50	None	50	—	58·0±6·4
Washed slices				
5·9	None	5	5·3±0·9*	4·1±1·3
0	None	5	14·3±1·2	17·0±1·8*

5·9mM-potassium. It was also shown that oxygen consumption in washed slices incubated in the absence of potassium (33·3±4·5 μmoles/g. of fresh tissue/30min.) was greater than in 5·9mM-potassium (23·4±1·1 μmoles/g. of fresh tissue/30min.); this is in contrast with the findings of Elliott & Bilodeau (1962).

Changes of intermediates with time in slices incubated in 5·9mM-potassium. In agreement with previous workers (McIlwain, Buchel & Cheshire, 1951), the tissue contents of ATP and creatine phosphate increased throughout the incubation (Table 2). It was also shown that ADP and AMP decreased with time, though the changes were not significant after 5min. incubation. Of the glycolytic intermediates measured, only fructose diphosphate and 2-phosphoglycerate showed statistically significant changes during incubation. Fructose diphosphate amounts rose to a maximum between 5 and 15min. (1300mμmoles/g. dry wt.) and decreased to 20% of this figure by 60min. (Table 2). The amount of 2-phosphoglycerate increased between 15 and 30min. Increasing the concentration of glucose in the medium from 5 to 50mM had no effect on the hexose phosphates, creatine phosphate or ATP after 5 or 15min. incubation; however, after 30min. incubation the higher concentration of glucose resulted in lower intracellular concentrations of fructose diphosphate and α-glycerophosphate.

Thus, in agreement with an earlier conclusion (Rolleston & Newsholme, 1967), 30min. incubation in 5·9mM-potassium was concluded to be sufficient time for cerebral cortex slices to reach a steady state. It was for this reason that additions of cyanide, glutamate, aspartate and ouabain to the incubations were made after 30min. preincubation, and slices were removed 7 or 30min. later.

Effects of cyanide on intermediates of glycolysis. In agreement with previous workers (see McIlwain, 1966), cyanide was found to decrease the intracellular concentrations of ATP and creatine phosphate (Table 3). The concentrations of inorganic phosphate in both medium and tissue extracts were also increased by the addition of cyanide; however, when expressed as intracellular concentrations the changes were not always statistically significant. It was also shown (Table 3) that cyanide increased the intracellular concentrations of AMP, α-glycerophosphate and lactate, and decreased those of citrate and the remaining phosphorylated intermediates of glycolysis except fructose diphosphate and the triose phosphates.

The effects of cyanide on fructose diphosphate and the triose phosphates were dependent on the concentration of glucose in the incubation medium (Table 3); in 5mM-glucose, cyanide caused the concentrations of these intermediates to decrease; in 50mM-glucose (or 100mM-glucose) their concentrations were increased by the presence of cyanide.

In slices incubated in 5mM-glucose, cyanide apparently caused glucose to disappear from the intracellular compartment (Table 3).

Table 2. *Amounts of intermediates in cerebral cortex slices after varying periods of incubation*

Slices were incubated for the times indicated in 3 ml. of medium containing 5·9 mM-potassium and 5 mM-glucose, and were then frozen as described in the text. Unincubated slices were frozen at the start of the incubation. Results are given as means ± S.D. of four independent observations.

Intermediates	Incubation period (min.)	Amounts (mμmoles/g. dry wt.)				
		0	5	15	30	60
Glucose 6-phosphate		170 ± 100	520 ± 60*	420 ± 80	525 ± 40	645 ± 105
Fructose diphosphate		560 ± 175	470 ± 80	1300 ± 100*	440 ± 150*	250 ± 100
α-Glycerophosphate		825 ± 185	845 ± 150	700 ± 60	850 ± 450	700 ± 200
3-Phosphoglycerate		780 ± 195	890 ± 100	740 ± 60	900 ± 250	700 ± 100
2-Phosphoglycerate		50 ± 10	65 ± 20	45 ± 5	115 ± 25*	115 ± 35
Phosphoenolpyruvate		200 ± 70	235 ± 65	230 ± 100	250 ± 30	225 ± 75
Citrate		1700 ± 900	1920 ± 400	2350 ± 500	2400 ± 500	3500 ± 1000
Creatine phosphate		1830 ± 1000	10900 ± 750*	13400 ± 450*	15600 ± 100*	17300 ± 150*
Adenosine triphosphate		3350 ± 350	5400 ± 150*	6750 ± 750*	7600 ± 650	7800 ± 500
Adenosine diphosphate		7450 ± 1350	2800 ± 350*	2420 ± 450	2160 ± 850	2010 ± 550
Adenosine monophosphate		6900 ± 750	805 ± 400*	525 ± 300	320 ± 150	310 ± 150

* ($P < 0·01$) Significance of difference from preceding figure.

Effects of potassium on intermediates of glycolysis. In agreement with previous workers (McIlwain, 1952), increasing the concentration of potassium in the medium in which cerebral cortex slices were incubated decreased the intracellular concentrations of ATP and creatine phosphate (Table 3). The concentrations of inorganic phosphate in both medium and tissue extracts were also increased by the increased concentration of potassium; however, intracellular concentrations of inorganic phosphate were not altered by incubation in 50 mM-potassium (Table 3). It was also shown that incubation of cerebral cortex slices in 50 mM-potassium caused decreased intracellular concentrations of ADP, the hexose monophosphates, 3-phosphoglycerate and phosphoenolpyruvate and increased concentrations of AMP.

The effects of 50 mM-potassium on some glycolytic intermediates were dependent on the concentration of glucose in the incubation medium (Table 3). In 5 mM-glucose, increasing the concentration of potassium from 5·9 to 50 mM caused decreased intracellular concentrations of fructose diphosphate, triose phosphates and α-glycerophosphate; in 50 mM-glucose, increasing the concentration of potassium caused increased concentrations of these intermediates. In slices incubated in 5 mM-glucose and 50 mM-potassium, glucose could not be detected in the intracellular compartment.

The effects of the absence of potassium were investigated with washed slices (see the Materials and Methods section). The intracellular concentrations of intermediates in washed slices incubated in the absence of potassium were compared with those of similar slices in 5·9 mM-potassium (Table 4). The absence of potassium caused lower concentrations of ATP, creatine phosphate, glucose 6-phosphate and α-glycerophosphate and higher concentrations of fructose diphosphate, triose phosphates, 3-phosphoglycerate, phosphoenolpyruvate, ADP, AMP and creatine (Table 4).

Effects of glutamate and aspartate on intermediates. The addition of glutamate to cerebral cortex slices incubated in 5·9 mM-potassium caused decreased tissue concentrations of creatine phosphate and ATP (McIlwain, 1952; Woodman & McIlwain, 1961; Rose, 1965), and asparatate was shown to have the same action (Table 3). In addition, it was shown that both glutamate and aspartate caused significantly increased concentrations of AMP without affecting ADP.

Both glutamate and aspartate caused significantly decreased concentrations of glucose 6-phosphate, fructose diphosphate and phosphoenolpyruvate (Table 3). Aspartate also decreased the α-glycerophosphate concentration, and glutamate decreased the concentration of citrate. In contrast with observations made with cyanide and 50 mM-potassium it was not possible to increase the concentration of fructose diphosphate in the presence of glutamate by increasing the glucose concentration to 50 mM.

Effects of ouabain on intermediates of glycolysis. Addition of high concentrations of ouabain to cerebral cortex slices incubated in 5·9 mM-potassium caused decreased concentrations of ATP and creatine phosphate (Rose, 1965; Swanson & McIlwain, 1965); 0·1 mM-ouabain was also shown to increase the concentration of AMP, and decrease those of glucose, glucose 6-phosphate, α-glycerophosphate and phosphoenolpyruvate (Table 3).

The most significant changes caused by addition

Table 3. *Effects of cyanide, potassium, glutamate, aspartate and ouabain on intracellular concentrations of glycolytic intermediates in guinea-pig cerebral cortex slices*

Intracellular concentrations of intermediates were measured as described in the Materials and Methods section. Results are expressed as means ± S.D. of four independent observations; the figures for the control condition (slices incubated in 5·9 mM-potassium and 5 or 50 mM-glucose) represent a typical mean of four incubations. Negative values for glucose concentrations indicate that the amount of glucose found in the tissue extracts was less than could be accounted for by the inulin space of the tissue. Conversion factors are described in the Materials and Methods section. * ($P < 0·01$), ** ($0·01 < P < 0·05$), Significance of differences from control slices incubated at the same time.

	Concn. of intermediates (mμmoles/ml. of intracellular fluid)								
Additions to incubation	Control		Cyanide		Potassium (50 mM)		Glutamate (5 mM)	Aspartate (5 mM)	Ouabain (0·1 mM)
Concn. of glucose (mM)	5	50	5	50	5	50	5	5	5
Intermediates									
Glucose	1280 ± 400	—	−160 ± 310*		−57 ± 88*		900 ± 460	800 ± 330	480 ± 380**
Glucose 6-phosphate	255 ± 38	252 ± 48	43 ± 26*	92 ± 25*	65 ± 22*	149 ± 16*	169 ± 17*	157 ± 14*	109 ± 28*
Fructose 6-phosphate	56 ± 16	68 ± 15	14 ± 10*	38 ± 16*	14 ± 4*	58 ± 20	33 ± 7	35 ± 12	24 ± 8
Fructose diphosphate	146 ± 33	93 ± 25**	39 ± 14*	130 ± 21	49 ± 19*	264 ± 65*	72 ± 16*	69 ± 28*	510 ± 220*
Triose phosphates	92 ± 28	66 ± 19	45 ± 15*	112 ± 26	46 ± 21*	205 ± 46*	79 ± 14	48 ± 17	343 ± 58*
α-Glycerophosphate	183 ± 43	102 ± 36*	590 ± 65*	465 ± 91*	104 ± 17*	166 ± 14*	135 ± 26	94 ± 25*	78 ± 4*
3-Phosphoglycerate	283 ± 72	265 ± 63	113 ± 36*	124 ± 65	114 ± 50*	184 ± 58	364 ± 91	279 ± 77	194 ± 59
2-Phosphoglycerate	26 ± 28	38 ± 19	14 ± 17	16 ± 8*	22 ± 5	19 ± 19	7 ± 9	14 ± 13	20 ± 15
Phosphoenolpyruvate	95 ± 25	58 ± 8	33 ± 10*	31 ± 8*	25 ± 5*	50 ± 26	25 ± 25*	29 ± 31*	35 ± 20*
Pyruvate	95 ± 32	98 ± 18	76 ± 35	88 ± 32	87 ± 26	92 ± 16	67 ± 55	120 ± 60	69 ± 40
Lactate	2560 ± 430	2380 ± 520	7020 ± 1830*	8380 ± 1320*	4250 ± 810*	4850 ± 550*	2160 ± 800	1940 ± 220	2660 ± 360
Citrate	1380 ± 280	900 ± 300	740 ± 120*	560 ± 220*	1160 ± 150	1240 ± 180	585 ± 230*	740 ± 195*	870 ± 84
Creatine phosphate	6180 ± 310	5530 ± 360	480 ± 180*	360 ± 110*	679 ± 110*	950 ± 470*	2520 ± 340*	2420 ± 415*	2300 ± 240*
Adenosine triphosphate	2980 ± 180	2630 ± 155	820 ± 230*	610 ± 230*	840 ± 85*	1130 ± 180*	1740 ± 150*	1750 ± 140*	1500 ± 130*
Adenosine diphosphate	695 ± 105	630 ± 180	640 ± 78	590 ± 90	555 ± 72*	575 ± 72	700 ± 52	660 ± 78	780 ± 70
Adenosine monophosphate	125 ± 15	120 ± 25	350 ± 52*	380 ± 90*	241 ± 25*	180 ± 42*	213 ± 26**	208 ± 14**	220 ± 11*
Creatine	4820 ± 1520	4950 ± 1780	4200 ± 1200	4680 ± 1050	4530 ± 1000	4730 ± 1300	6110 ± 540	5900 ± 890	6790 ± 1240
Phosphate	7630 ± 1660	7520 ± 1420	9510 ± 1240	10030 ± 1130*	6790 ± 1230	6580 ± 1200	7010 ± 1720	6950 ± 1410	7390 ± 1200
Conversion factor	2·8	2·8	3·8	3·8	4·2	4·2	3·8	3·8	3·6

Table 4. *Effects of potassium on intermediates in washed cerebral cortex slices*

Slices were washed for 1 hr. at 0° in 6 ml. of potassium free medium to decrease their content of potassium. They were then transferred to 3 ml. of incubation medium containing 5 mM-glucose and the potassium ion concentrations indicated. Incubation was for 30 min. at 38° before freezing as described in the text. Results are given as means ± S.D. of four independent observations. Conversion factors are as in the text. * $P < 0.01$; ** $0.01 < P < 0.05$.

Intermediates	Concn. of K^+ in medium ...	Concentrations (mμmoles/ml. of intracellular water) 0	5·9
Glucose		3060 ± 650	3610 ± 700
Glucose 6-phosphate		171 ± 20	254 ± 16*
Fructose 6-phosphate		56 ± 22	56 ± 30
Fructose diphosphate		297 ± 36	70 ± 30*
Triose phosphates		127 ± 29	26 ± 10*
α-Glycerophosphate		115 ± 13	152 ± 16*
3-Phosphoglycerate		382 ± 32	246 ± 28*
2-Phosphoglycerate		31 ± 11	42 ± 20
Phosphoenolpyruvate		122 ± 20	70 ± 38**
Pyruvate		76 ± 27	51 ± 31
Lactate		2260 ± 230	2200 ± 254
Citrate		590 ± 176	450 ± 62
Creatine phosphate		1670 ± 200	2970 ± 160*
Adenosine triphosphate		1760 ± 54	2350 ± 46*
Adenosine diphosphate		720 ± 83	480 ± 100*
Adenosine monophosphate		194 ± 47	66 ± 40*
Creatine		4300 ± 530	3200 ± 143*
Phosphate		2720 ± 430	2380 ± 410
Conversion factor		2·8	2·5

of 0·1 mM-ouabain involved the intracellular concentrations of fructose diphosphate and the triose phosphates (Table 3); these appeared to increase linearly with time after addition of the inhibitor, reaching 1·4 and 0·7 mM respectively (14–15-fold increase) after 30 min. As there was no corresponding increase in 3-phosphoglycerate, it is concluded that addition of 0·1 mM-ouabain to guinea-pig cerebral cortex slices caused partial inhibition of either glyceraldehyde 3-phosphate dehydrogenase or phosphoglycerate kinase or both. As this accumulation of intermediates was not observed in washed slices incubated in the absence of potassium or in fresh slices in the presence of cyanide, these effects of ouabain are concluded not to be dependent on inhibition of the active transport system for the alkali metal ions (Rolleston & Newsholme, 1966*b*).

The addition of 0·1 mM-ouabain to cerebral cortex slices also affected the relation between the lactate/pyruvate and the α-glycerophosphate/dihydroxyacetone phosphate couples. Under all the other conditions studied, the quotient of lactate/pyruvate ÷ α-glycerophosphate/dihydroxyacetone phosphate in cerebral cortex slices lay between 6·8 and 66·0; in the presence of 0·1 mM-ouabain, this quotient reached a value of 560 after 30 min. incubation. No explanation for any of these effects of 0·1 mM-ouabain is offered.

The only significant effect (on intracellular concentrations) of the addition of μM-ouabain to cerebral cortex slices was an increased intracellular concentration of glucose; this occurred without a significant change in the extracellular concentration.

DISCUSSION

Identification of 'non-equilibrium' reactions. The first stage in locating reactions at which metabolic pathways may be controlled is to identify those that are far from equilibrium ('non-equilibrium'). To this end, mass-action ratios (Γ) for the reactions of glycolysis have been compared with their apparent equilibrium constants (K') (Table 5). The reactions catalysed by phosphoglucose isomerase, phosphoglycerate mutase, enolase, adenylate kinase and creatine phosphokinase were close to equilibrium, and those catalysed by phosphofructokinase and pyruvase kinase were far from equilibrium under all conditions. The classification of hexokinase and the system permitting the entry of glucose into the intracellular fluid as 'equilibrium' or 'non-equilibrium' depends on the intracellular glucose concentration. When this was significantly greater than zero (Table 3), hexokinase was clearly catalysing a 'non-equilibrium' reaction ($\Gamma = 0.01 - 0.23$, Table 5) and the glucose entry system was close to equilibrium ($\Gamma = 0.18 - 0.92$, Table 5). However, when intracellular glucose concentrations were low (e.g. in slices incubated in the presence of cyanide or 50 mM-potassium, Table 3), the glucose entry system was concluded to be far from equilibrium ($\Gamma = 0$, Table 5), and the hexokinase reaction may have been close to equilibrium (Γ very large, ∞ in Table 5).

Mass-action ratios for the remaining reactions of glycolysis cannot be calculated directly from measurements made in this work; 1,3-diphosphoglycerate, $NADH_2$ and NAD were not measured, and dihydroxyacetone phosphate and glyceraldehyde 3-phosphate were measured together as triose phosphates. However, if it is assumed that the reaction catalysed by triose phosphate isomerase is at equilibrium (Lowry & Passonneau, 1964) the measurements of triose phosphate concentrations can be used to calculate the concentrations of glyceraldehyde 3-phosphate and dihydroxyacetone phosphate. Similarly, assumption of equilibrium at lactate dehydrogenase permits calculation of the ratio of $NADH_2$/NAD from the ratio of lactate/pyruvate. With these assumptions, the mass-action ratios for the aldolase reaction and for the combination of the reactions catalysed by gly-

Table 5. *Comparison between mass-action ratios and apparent equilibrium constants for the reactions of glycolysis*

Mass-action ratios were calculated from the concentrations of intermediates in Tables 3 and 4. The general reaction considered was A+B⇌C+D in the direction of flow through glycolysis, or in the direction of ATP synthesis for the two non-glycolytic enzymes, and the mass-action ratio was calculated by using the equation $\Gamma=[C][D]/[A][B]$. The apparent equilibrium constants for enzymes assumed to be at equilibrium (see text) were 0·036 for triose phosphate isomerase (in the direction of glyceraldehyde 3-phosphate formation; Lowry & Passonneau, 1964) and $1{\cdot}89\times10^4$ for lactate dehydrogenase (in the direction of lactate formation; Hohorst, Kreutz & Bücher, 1959). Apparent equilibrium constants quoted were taken from these references: (1) Krebs & Kornberg (1957) using $\Delta G=-RT\ln K'$; (2) Hess (1963); (3) Lowry & Passonneau (1964); (4) Eggleston & Hems (1952); (5) Cori, Velick & Cori (1950) for glyceraldehyde 3-phosphate dehydrogenase and Krebs & Kornberg (1957) for phosphoglycerate kinase; (6) Gilbert (1965); (7) Kuby & Noltmann (1962).

Enzymes	Apparent equilibrium constants (K')	Mass-action ratios observed (Γ)	Units of K' and Γ
Glucose entry	$\geqslant1$[6]	0·18–0·92 and 0*	—
Hexokinase	3900[1] 5500[2]	0·01–0·23 and ∞*	—
Phosphoglucoisomerase	0·45[1] 0·47[2]	0·19–0·41	—
Phosphofructokinase	910[1] 1200[2]	0·06–10·7	—
Aldolase	130[1] 68[2] 90[3]	0·03–12·4	μM
Glyceraldehyde 3-phosphate dehydrogenase plus phosphoglycerate kinase	183[1] 310[1] 1550[5]	8·6–1320	M^{-1}
Phosphoglycerate mutase	0·18[1] 0·17[2] 0·10[3]	0·02–0·19	—
Enolase	2·8[1] 1·4[2] 4·6[3]	1·7–3·9	—
Pyruvate kinase	20000[1] 15000[2]	1·5–11·0	—
Creatine phosphokinase	5·5–24[3]	3·3–13·4	—
Adenylate kinase	0·44[4]	0·49–0·83	—

* See text.

ceraldehyde 3-phosphate dehydrogenase and phosphoglycerate kinase may be calculated from the measurements made.

The lactate dehydrogenase reaction was chosen in preference to the α-glycerophosphate dehydrogenase reaction because of the high activity of α-glycerophosphate oxidase in brain (Sacktor, Packer & Estabrook, 1959); this, together with the low activity of α-glycerophosphate dehydrogenase in brain (Lowry & Passonneau, 1964), provides an explanation for the observation that the quotients of lactate/pyruvate ÷ α-glycerophosphate/dihydroxyacetone phosphate observed in cerebral tissue are higher than the equilibrium value 1·6 (see Zebe, Delbrück & Bücher, 1959).

The mass-action ratios for the aldolase reaction were between seven- and 3000-fold less than the apparent equilibrium constant (Table 5). It has been suggested that the cause for this apparent disequilibrium is that a large portion of the fructose diphosphate is not available to aldolase (Lowry & Passonneau, 1964); disequilibrium is maintained even when the rate of flow through glycolysis is very low. It is thus concluded that aldolase catalyses an 'equilibrium' reaction in cerebral cortex slices.

The calculated mass-action ratios obtained for the reaction catalysed by the combination of glyceraldehyde 3-phosphate dehydrogenase with phosphoglycerate kinase ranged from 8·6 to 1320, whereas the apparent equilibrium constants obtained from the literature ranged from 183 to 1550 (Table 5). Since the lactate dehydrogenase and triose phosphate isomerase reactions were assumed to be at equilibrium in order to calculate the mass-

action ratios, and since it is probable that the intracellular inorganic phosphate measured is at least partly bound in the tissue, it was not possible from the available evidence to classify this reaction as 'equilibrium' or 'non-equilibrium'. If the reaction is assumed to be 'non-equilibrium', glyceraldehyde 3-phosphate dehydrogenase probably causes the disequilibrium, as it is considerably less active in brain than phosphoglycerate kinase (Lowry & Passonneau, 1964).

Identification of regulatory enzymes. From the preceding discussion it has been concluded that the reactions catalysed by hexokinase, phosphofructokinase and pyruvate kinase were generally 'non-equilibrium'. In addition, the glyceraldehyde 3-phosphate dehydrogenase reactions might have been 'non-equilibrium'.

Enzymes catalysing 'non-equilibrium' reactions may be tested for their regulatory significance by the principle suggested by Krebs (1957); if the concentration of substrate for a 'non-equilibrium' reaction changes in the opposite direction to the change in rate of flow through the reaction, then the enzyme catalysing that reaction must be regulatory. The information relevant to this principle is summarized in Table 6. From this summary, it is concluded that hexokinase, phosphofructokinase and pyruvate kinase are regulatory. Also if glyceraldehyde 3-phosphate dehydrogenase catalyses a 'non-equilibrium' reaction, then it may also be concluded to be a regulatory enzyme.

Mechanism of control over glycolysis. Theories of control of a metabolic pathway depend on knowledge of the properties of its regulatory enzymes. Since the properties of the enzymes of glycolysis have been thoroughly investigated, the concentrations of possible regulatory compounds were measured in the cerebral cortex slices together with the intermediates of the pathway. The mechanisms of control over glycolysis proposed in this paper are consistent with the known properties of the regulatory enzymes and with observed changes of intracellular concentrations of regulatory intermediates. To simplify the discussion, only the mechanism of increasing the rate of glycolysis will be discussed.

It is suggested that hexokinase and phosphofructokinase together form a regulatory system. ATP inhibits phosphofructokinase, and AMP relieves this inhibition (Lardy & Parks, 1956; Mansour & Mansour, 1962; Passonneau & Lowry, 1962, 1963). The activity of adenylate kinase which catalyses an 'equilibrium' reaction in cerebral cortex slices (Table 5) and the relative proportions of ATP and AMP in the tissue together permit a small fractional decrease in the concentration of ATP to cause a large fractional increase in the concentration of AMP (Krebs, 1964). Thus any condition decreasing the concentration of ATP would tend to increase the activity of phosphofructokinase with the changes in AMP concentration acting to amplify the effects of ATP. The resultant increased activity of phosphofructokinase

Table 6. *Summary of statistically significant changes in rates of flow through glycolysis and concentrations and amounts of substrates for the 'non-equilibrium' reactions*

All changes are relative to the control slices incubated in 5·9mM-potassium medium; + and − indicate an increase or decrease respectively. Amounts of intermediates (mμmoles/g. dry wt.) may be calculated from concentrations (mμmoles/ml. of intracellular fluid) by using the conversion factors in Tables 3 and 4. Changes in amounts are included in this summary as the swelling of cerebral cortex slices caused by some of the conditions of incubation could have caused spurious reductions in measured intracellular concentrations. (−) indicates that the significant changes were observed only in intermediates in equilibrium with the substrates for the 'non-equilibrium' reactions. Results are grouped as follows: (*a*) experiments in which fresh slices were incubated in 5mM-glucose; (*b*) experiments in which fresh slices were incubated in 50mM-glucose; (*c*) experiments with washed slices incubated in 5mM-glucose.

	Substrates ...	...	Glucose		Fructose 6-phosphate (glucose 6-phosphate)		Triose phosphates (fructose diphosphate)		Phosphoenolpyruvate (3-phosphoglycerate)	
Group	Additions	Rates	Concn.	Amount	Concn.	Amount	Concn.	Amount	Concn.	Amount
(*a*)	Cyanide (3mM)	+	−	−	−	−	−	(−)	−	(−)
	Glutamate (5mM)	+			(−)		(−)		−	
	Aspartate (5mM)	+			(−)		(−)		−	
	Ouabain (0·1mM)	+			−	−	+	+	−	
	Ouabain (1μM)	−	+	+						
	Potassium (50mM)	+	−	−	−	−	−	(−)	−	−
(*b*)	Cyanide (3mM)	+			−	−	+	+	−	
	Potassium (50mM)	+			−		+	+		
(*c*)	Potassium (0mM)	+			−	−	+	+	+	+

would tend to decrease the concentration of fructose 6-phosphate, which is in equilibrium with glucose 6-phosphate, an inhibitor of hexokinase (Weil-Malherbe & Bone, 1951). Thus the activity of hexokinase is linked to that of phosphofructokinase by a feedback-inhibition mechanism. Since the inhibition of hexokinase by glucose 6-phosphate is non-competitive with respect to glucose (Weil-Malherbe & Bone, 1951; see Walker, 1966) excess of glucose cannot increase the rates of glycolysis. However, the inhibition of phosphofructokinase by ATP can be overcome by excess of fructose 6-phosphate (Passonneau & Lowry, 1962, 1963; Underwood & Newsholme, 1965); thus supplies of glucose 6-phosphate other than from hexokinase may be metabolized via phosphofructokinase. This provides a very flexible regulatory system.

If glyceraldehyde 3-phosphate dehydrogenase catalyses a 'non-equilibrium' reaction in cerebral cortex slices, it may form a regulatory system with pyruvate kinase similar to that suggested for hexokinase and phosphofructokinase. ATP inhibits pyruvate kinase (McQuate & Utter, 1959; Lowry & Passonneau, 1964), whose substrate, phosphoenolpyruvate, is linked to 1,3-diphosphoglycerate, a product inhibitor of glyceraldehyde 3-phosphate dehydrogenase (Velick & Furfine, 1963) by 'equilibrium' reactions. Changes in pyruvate kinase activity could affect glyceraldehyde 3-phosphate dehydrogenase activity through changes in the concentration of 1,3-diphosphoglycerate. The effects of the ratio ATP/ADP on the ratio 1,3-diphosphoglycerate/3-phosphoglycerate, due to the proposed 'equilibrium' at the phosphoglycerate kinase reaction, could act to amplify the effects of ATP control over pyruvate kinase. However, this postulated control mechanism is possible only if glyceraldehyde 3-phosphate dehydrogenase catalyses a 'non-equilibrium' reaction.

Rate-limiting systems for glycolysis in cerebral cortex slices. Since fructose diphosphate relieves the inhibition of phosphofructokinase by ATP (Passonneau & Lowry, 1962), there can be no direct feedback mechanism by which the enzymes metabolizing fructose diphosphate can control the activity of phosphofructokinase. Therefore the rate of glucose uptake by cerebral cortex slices must be limited at a point before the formation of fructose diphosphate. Thus the two possible rate-limiting systems for glycolysis are the hexokinase–phosphofructokinase system and the system permitting the entry of glucose into the tissue.

In slices incubated in cyanide or in the presence of 50mM-potassium increasing the concentration of glucose in the medium (from 5mM) caused increased rates of glucose uptake and lactate formation (Table 1) and also increased the intracellular concentrations of fructose diphosphate and the triose phosphates (Table 3). Also in slices incubated in 5mM-glucose under these conditions intracellular concentrations of glucose were very low. These observations lead to the conclusions that, under these conditions, glucose metabolism was limited by the rate of entry of glucose into the tissue. However, in the remaining conditions of incubation, when glucose could be demonstrated in the intracellular space, increasing the concentration of glucose in the incubation medium had no effect on rates of glucose metabolism and also did not increase the concentrations of intermediates. Under such conditions, the hexokinase–phosphofructokinase system must be regarded as rate-limiting to glycolysis.

Although glucose entry has been shown to be rate-limiting, it is not known if it is regulatory, i.e. if it can change its activity independently of substrate. This has not been tested in this work as no two conditions in which it was rate-limiting were compared.

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REFERENCES

Ashford, C. A. & Dixon, K. C. (1935). *Biochem. J.* **29**, 157.
Berenblum, J. & Chain, E. (1938). *Biochem. J.* **32**, 295.
Bergmeyer, H.-U. (Ed.) (1963). *Methods of Enzymatic Analysis.* New York: Academic Press Inc.
Bücher, T. & Rüssmann, W. (1964). *Angew. Chem. (int. Ed.)* **3**, 426.
Chance, B., Holmes, W., Higgins, J. & Connelly, C. M. (1958), *Nature, Lond.*, **182**, 1190.
Cori, C. F., Velick, S. F. & Cori, G. T. (1950). *Biochim. biophys. Acta*, **4**, 160.
Eggleston, L. V., & Hems, R. (1952). *Biochem. J.* **52**, 156.
Elliott, K. A. C. & Bilodeau, F. (1962). *Biochem. J.* **84**, 421.
Gilbert, H. (1965). *Nature Lond.* **205**, 87.
Goldberg, N. D., Passonneau, J. V. & Lowry, O. H. (1966). *J. biol. Chem.* **241**, 3997.
Hess, B. (1963). In *Control Mechanisms in Respiration and Fermentation*, p. 333. Ed. by Wright, B. New York: Ronald Press Co.
Hohorst, H. J., Kreutz, F. H. & Bücher, T. (1959). *Biochem. Z.* **332**, 18.
Krebs, H. A. (1957). *Endeavour*, **16**, 125.
Krebs, H. A. (1964). *Proc. Roy. Soc.* B, **159**, 545.
Krebs, H. A. & Henseleit, G. (1932). *Hoppe-Seyl. Z.* **210**, 33.
Krebs, H. A. & Kornberg, H. L. (1957). *Ergebn. Physiol.* **49**, 212.
Kuby, S. A. & Noltmann, E. A. (1962). In *The Enzymes*, vol. 6, p. 515. Ed. by Boyer, P. D., Lardy, H. A. & Myrbäck, K. New York: Academic Press Inc.
Lardy, H. A. & Parks, R. E. (1956). In *Enzymes: Units of Biological Structure and Function*, p. 584. Ed. by Gaebler, D. H. New York: Academic Press Inc.

Lowry, O. H. & Passonneau, J. V. (1964). *J. biol. Chem.* **239**, 31.

Lowry, O. H., Passonneau, J. V., Hasselberger, F. X. & Schulz, D. W. (1964). *J. biol. Chem.* **239**, 18.

McIlwain, H. (1952). *Biochem. J.* **52**, 289.

McIlwain, H. (1966). *Biochemistry of the Central Nervous System.* 3rd ed., pp. 64, 124. London: J. and A. Churchill Ltd.

McIlwain, H., Buchel, L. & Cheshire, J. D. (1951). *Biochem. J.* **48**, 12.

McQuate, J. T. & Utter, M. F. (1959). *J. biol. Chem.* **234**, 2151.

Mann, P. F. E., Trevelyan, W. E. & Harrison, J. S. (1958). In *Recent Studies in Yeast and their Significance in Industry*, p. 68. London: Society of Chemical Industry.

Mansour, T. E. & Mansour, J. M. (1962). *J. biol. Chem.* **237**, 629.

Martin, J. B. & Doty, D. M. (1949). *Analyt. Chem.* **21**, 965.

Morrison, J. F. (1954). *Biochem. J.* **56**, 99.

Pappius, H. M. & Elliott, K. A. C. (1956). *Canad. J. Biochem. Physiol.* **34**, 1007.

Passonneau, J. V. & Lowry, O. H. (1962). *Biochem. biophys. Res. Commun.* **7**, 10.

Passonneau, J. V. & Lowry, O. H. (1963). *Biochem. biophys. Res. Commun.* **13**, 372.

Rolleston, F. S. (1966). D.Phil. Thesis: University of Oxford.

Rolleston, F. S. & Newsholme, E. A. (1966*a*). *Biochem. J.* **100**, 64P.

Rolleston, F. S. & Newsholme, E. A. (1966*b*). *Biochem. J.* **101**, 41P.

Rolleston, F. S. & Newsholme, E. A. (1967). *Biochem. J.* **104**, 519.

Rose, S. P. R. (1965). *Biochem. Pharmacol.* **14**, 589.

Sacktor, B., Packer, L. & Estabrook, R. W. (1959). *Arch. Biochem. Biophys.* **80**, 68.

Schwarz, A. (1962). *Biochem. Pharmacol.* **11**, 389.

Siebert, G. (1963). In *Methods of Enzymatic Analysis*, p. 318. Ed. by Bergmeyer, H.-U. New York: Academic Press Inc.

Swanson, P. D. & McIlwain, H. (1965). *J. Neurochem.* **12**, 877.

Thorn, W., Scholl, H., Pfleiderer, G. & Mueldener, B. (1958). *J. Neurochem.* **2**, 150.

Underwood, A. H. & Newsholme, E. A. (1965). *Biochem. J.* **95**, 868.

Varon, S. & McIlwain, H. (1961). *J. Neurochem.* **8**, 262.

Velick, S. F. & Furfine, C. (1963). In *The Enzymes*, 2nd ed., vol. 7, p. 267. Ed. by Boyer, P. D., Lardy, H. A. & Myrbäck, K. New York: Academic Press Inc.

Walker, D. G. (1966). In *Essays in Biochemistry*, vol. 2, p. 33. Ed. by Campbell, P. N. & Greville, G. D. London and New York: Academic Press Inc.

Weil-Malherbe, H. (1938). *Biochem. J.* **32**, 2257.

Weil-Malherbe, H. & Bone, A. D. (1951). *Biochem. J.* **49**, 339.

Wollenberger, A. (1947). *J. Pharmacol.* **91**, 39.

Woodman, R. J. & McIlwain, H. (1961). *Biochem. J.* **81**, 83.

Zebe, E., Delbrück, A. & Bücher, T. (1959). *Biochem. Z.* **331**, 254.